INHIBITION OF TRANSLATION IN RETICULOCYTE LYSATE BY THE MYCOTOXIN PATULIN

F. HATEY and P. GAYE+

Laboratoire de Pharmacologie-Toxicologie, INRA, 180 Chemin de Tournefeuille, 31300, Toulouse and
+*Laboratoire de Physiologie de la lactation, CNRZ, 78350 Jouy en Josas, France*

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1. Introduction

Patulin, a mycotoxin produced by various fungi from *Penicillium* and *Aspergillus* species, is toxic to microorganisms, cells and animals [1]. Different studies have demonstrated its inhibitory properties towards incorporation of labelled precursors into DNA, RNA and proteins of cultured cells [2,3], leucine uptake [4] and glycine transport [5] in reticulocytes. Recently, it has been demonstrated that patulin inhibits in vitro transcription [6], but the effect of this drug on translation has not been investigated.

It has been suggested [7] that patulin exerts its toxicity by interacting with SH groups and confirmed [8] that the mycotoxin reacts with glutathione so that this compound could no longer be detected.

The present work reports the effects of patulin on in vitro translation in a rabbit reticulocyte cell-free system, and indicates that the observed inhibition could result from an interaction of the drug with SH groups from various factors involved in protein synthesis.

2. Materials and methods

Reticulocytes from phenylhydrazine-treated rabbits were collected and repeatedly washed by centrifugation in an isotonic buffer. Lysis was achieved by adding ice-cold distilled water to the cells. The lysate was stored in small aliquots at −80°C until used.

Final concentrations in standard assay were 80 mM KCl, 2 mM $Mg(CH_3COO)_2$, 0.2 mM GTP, 1 mM ATP, 0.1 mM each of 19 amino acids (minus leucine). [^{3}H] Leucine (30 Ci/mmol, CEA) was added at final conc. 0.34 μM, phosphocreatine at 24 mM and creatine kinase in sufficient amounts. Hemin was 20 μM. The final volumes were 50 μl, 150 μl or 200 μl containing 20 μl, 60 μl or 80 μl of lysate, respectively.

After a 90 min incubation (unless otherwise stated) at 27°C, two 10 μl aliquots were diluted with 100 μl ice-cold distilled water and proteins were precipitated with 5% (w/v) trichloroacetic acid (TCA)-containing 1% unlabelled leucine. After 10 min at 90°C, the precipitates were collected on glass fiber filters (Schleicher and Schull no. 6 or Whatmann GF/C), washed twice with 5% TCA (containing unlabelled leucine as above) then with alcohol–ether (1:1, v/v), and dried. Radioactivity was measured in a scintillation mixture (PPO 4%, dimethyl POPOP 0.1% in toluene) with an Intertechnique (France) spectrometer.

Polysome profiles were obtained on exponential sucrose density gradients (0.3 M–1 M) made in a standard buffer (Tris–HCl 10 mM (pH 7.8); KCl 10 mM; $MgCl_2$, 1.5 mM). After incubation as indicated in the figures, the mixture (200 μl) was diluted with cold standard buffer (400 μl) and layered onto the gradients. Centrifugation was performed in a Beckman SW 41 Ti rotor at 40 000 rev./min during 60 min at 4°C. Fractions (0.4 ml) were collected using a density gradient fractionator (Model 640, ISCO), with continuous A_{254} recording (ISCO, model UA5). The fractions were diluted with distilled water (0.6 ml) and proteins were precipitated with 0.25 ml 50% (w/v) TCA and processed as above.

Patulin was isolated from cultures of *Byssochlamys nivea* and its purity was checked by thin-layer chromatography [9].

3. Results and discussion

Addition of patulin to a reticulocyte cell-free system resulted in an inhibition of leucine incorporation into proteins. The dose–response curve (fig.1) shows that doses $<10^{-4}$ M were not inhibitory and that maximal inhibition was obtained with 10^{-3}–10^{-2} M. The 50% inhibitory dose (ID_{50}) was about 3–6 $\times$ 10^{-4} M, a relatively high concentration if one compares it to cycloheximide whose ID_{50} in our conditions was about 3 $\times$ 10^{-7} M (data not shown). However, in vitro translation appears to be more sensitive to patulin than in vitro transcription: ID_{50} for the latter has been reported to be about 1.3 $\times$ 10^{-3} M [6]. In intact reticulocytes, 50% inhibition of amino acid transport is achieved by 2 $\times$ 10^{-4} M patulin [4,5], but cells in culture appear more sensitive: ID_{50} for transcription and translation in HeLa cells is about 10^{-5} M, and slightly lower (6 $\times$ 10^{-6} M) for replication [2]. In Chang liver cells, 1.7 $\times$ 10^{-5} M inhibits protein synthesis by 40% and RNA synthesis by 60% [3].

Experiments were designed to define which step of the translational process was inhibited. This was achieved by comparing patulin with specific inhibitors of either initiation (aurintricarboxylic acid (ATA) [10]) or elongation (cycloheximide and sparsomycin [11,12]) in two sets of experiments: time course incorporation of leucine and polysome sedimentation patterns (A_{254} profile and incorporated radioactivity distribution) on sucrose density gradients.

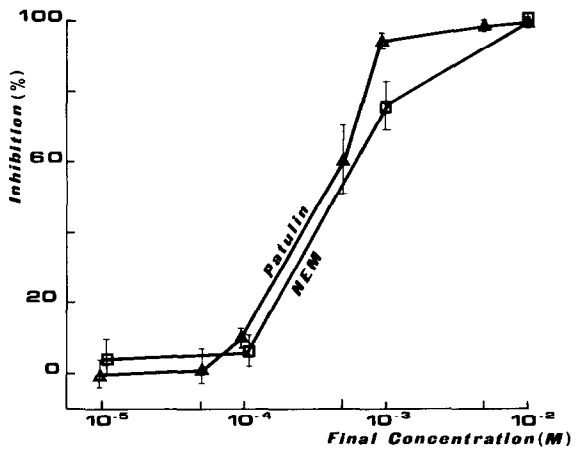


Fig.1. Dose–response curve of the inhibition produced by patulin (△) or NEM (□) on in vitro translation in a rabbit reticulocyte lysate. Results are the mean ± SEM of values obtained in 5 (patulin) or 6 (NEM) separate experiments.

The difference between inhibition of elongation or initiation is shown in time course incorporation experiments (fig.2A): when 10^{-5} M sparsomycin (or 10^{-2}–10^{-3} M cycloheximide) was added after 2 min incubation, there was no further incorporation;

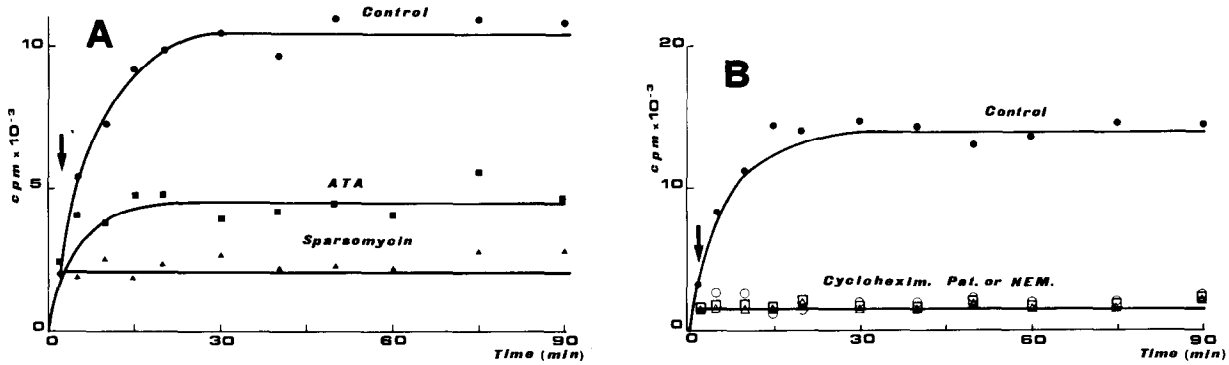
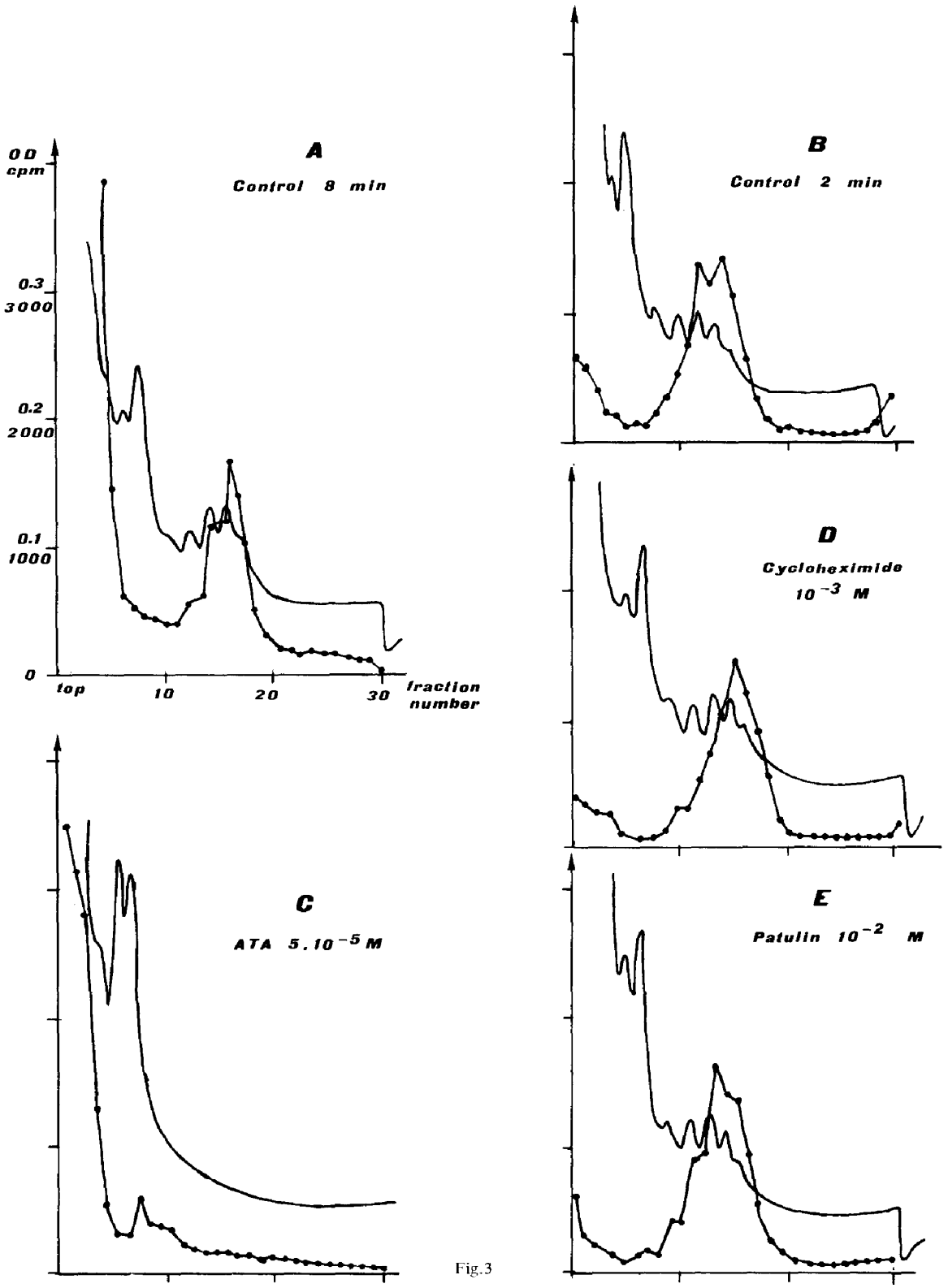


Fig.2. Effects of different drugs on time course incorporation of [^{3}H]leucine in a reticulocyte lysate. Drugs were added 2 min after the beginning of the incubation (arrow). Samples (10 μl) were removed at different times and processed as in section 2. (A): (●) control; (■) aurintricarboxylic acid (ATA) 5 $\times$ 10^{-5} M; (▲) sparsomycin 10^{-5} M. B: (●) control; (△) patulin 10^{-2} M; (○) NEM 10^{-2} M; (□) cycloheximide 10^{-2} M.


A
Control 8 min
OD
cpm
0.3
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B
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C
ATA 5.10⁻⁵ M
D
Cycloheximide
10⁻³ M
E
Patulin 10⁻² M

Fig.3

on the contrary, when 5×10^{-5} M ATA was added at that time, there was a lag before appearance of complete inhibition, which corresponds to the elongation, termination and release of the peptide chains already initiated at the time of addition of the inhibitor. When patulin was added in the same conditions (fig.2B) the incorporation of leucine was immediately stopped as in the case of cycloheximide or sparsomycin. However, when doses $<10^{-2}$ M were used, a lag before the appearance of the inhibition was observed. The same response was obtained with $\leqslant 10^{-4}$ M cycloheximide. This result agrees with an inhibitory effect of patulin on the elongation step.

This observation was confirmed by analysis of polysome sedimentation patterns on sucrose density gradients (fig.3). Inhibition of elongation by cycloheximide (10^{-3} M) results in a 'freezing' of the polysomes: absorbance profile as well as incorporated radioactivity distribution are similar to those of control at time of drug addition (fig.3, cf. D with B). Similar results were obtained with sparsomycin (data not shown). On the contrary, inhibition of initiation by ATA (5×10^{-5} M) results in polysome breakdown with release of the radioactivity in the supernatant (fig.3C). Patulin (10^{-2} M) gives results similar to those of cycloheximide (fig.3E). Thus our results show clearly that the mycotoxin interferes with the elongation mechanism; nevertheless, a possible action of patulin on the initiation step cannot be excluded.

Patulin has been suggested to exert its toxic activity by interacting with SH groups [7]. In order to test this hypothesis, we have compared the above reported effects of patulin with those of a widely used SH reagent, *N*-ethylmaleimide (NEM), in the same experimental conditions: no major difference appeared in either dose–response curve (fig.1), time course inhibition (fig.2B) or polysome sedimentation pattern and radioactivity distribution on sucrose density gradients (data not shown).

To further confirm the mechanism involving sulfhydryl groups, we investigated the effect of addition of extra SH groups, in the form of reduced glutathione (GSH), on the dose–response curve of either patulin or NEM assuming that reaction of these molecules with glutathione would reduce or suppress their inhibitory potency. Results of such experiments are shown in fig.4: we preincubated the drugs with either glutathione, in a molar concentration twice that of either drug, or lysate, and compared the results to those obtained when the inhibitor was preincubated alone. The preincubation of patulin or NEM with glutathione results in a reduction of inhibition. On the contrary, preincubation of patulin or NEM with lysate allows the inhibitory activity to develop fully, the addition of glutathione being unable to remove this inhibition.

This observation is compatible with a covalent binding of NEM and patulin with SH groups of either glutathione or active proteins in the reticulocyte lysate. Nevertheless, patulin seems to react less rapidly than NEM does, since after preincubation with GSH, it retained some inhibitory potency and NEM did not. Moreover, when preincubated alone, patulin seems to react poorly with GSH in presence of the other components as its dose–response curve is nearly unmodified. By contrast, NEM looses all its inhibitory potency.

The results reported here present the inhibitory properties of patulin towards translation: apart from an effect on amino acid transport across cell membranes [6] which can be, at least partly, responsible of the in vivo inhibition of protein synthesis, we have demonstrated a direct effect on the mechanism of translation. To what extent this inhibition accounts for the toxicity of the molecule remains to be determined. However, if the interaction of patulin with SH groups is involved in the effects reported here, we cannot assume that there is no other mechanism that may contribute to the inhibition of translation and, more generally, to the toxicity of this mycotoxin.

Fig.3. Polysome sedimentation pattern and distribution of radioactivity. Five identical reaction mixtures were incubated at 27°C for 2 min, then treated as follows: controls were either incubated 6 min longer (A) or immediately stopped (B); assays were incubated 6 min longer, following addition of either ATA (final conc. 5×10^{-5} M) (C); cycloheximide (final conc. 10^{-3} M) (D); or patulin (final conc. 10^{-2} M) (E). Sucrose gradients (0.1 M–1 M) were centrifuged and fractionated as in section 2. (——) A_{254}. (•—•) Incorporated radioactivity.

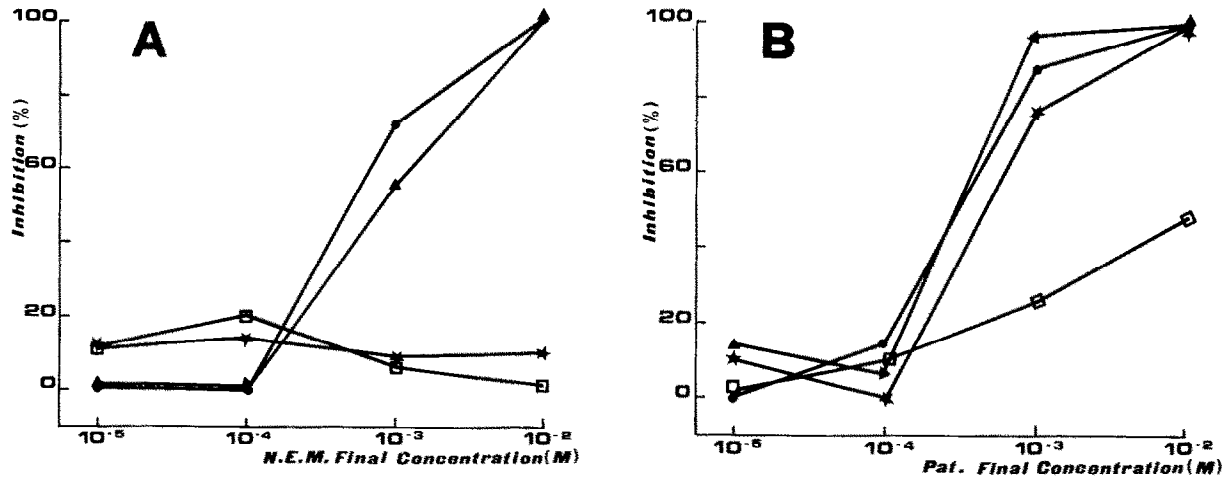

Fig.4. Effect of reduced glutathione (GSH) on NEM (A) or patulin (B) dose–response curve in different experimental conditions. A 5 min preincubation (27°C) was performed with NEM or patulin alone or with either lysate or GSH. Then, other components, already mixed ('Mix'), were added to ensure a final reaction mixture identical to the standard one. Incubation (90 min, 27°C) and further processing were conducted as usual. Experimental conditions were as follows: Preincubation of NEM (A) or patulin (B). (●) Alone; (*) alone; (▲) + lysate; (□) + GSH. 'Mix'. Salt, energy and amino acids (including [^{3}H]leucine): + hemin, lysate; + GSH, hemin, lysate; + GSH, hemin; + hemin, lysate.

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